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14. ABSTRACT

The specific aims of this project are to: 1) determine the efficacy, pharmacokinetics, toxicology and imaging capacity of RGD-targeted Nexil; and, 2) determine the ability of other ovarian cancer-specific targeting ligands to enhance the efficacy of Nexil. Substantial progress has been made on both of these specific aims. This progress report covers work done during the first 10 months of the grant. Significant achievements to date include: the design and synthesis of two different linkers to be used in the coupling with the RGD peptide to the Nexil polymer; the synthesis of cyclic RGD peptide using Fmoc strategy on solid support; the coupling of cyclic RGD to linkers, deprotection and documentation of purity; synthesis of a set of molecules containing 5, 15 and 30 RGD units per polyglutamylglutamate molecule; establishment of a fluorescence polarization assay for assessment of affinity of binding to soluble integrins; addition of a fluorochrome to RGD-linker-polymer to facilitate pharmacokinetic and tissue distribution studies; and, establishment of a strategy for the synthesis of Lyp-1 using a solid support strategy.

15. SUBJECT TERMS
Efficacy, pharmacokinetics, toxicology, imaging capacity

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Table of Contents

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Principal investigator: Stephen B. Howell, M.D.
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INTRODUCTION

The specific aims of this project are to: 1) determine the efficacy, pharmacokinetics, toxicology and imaging capacity of RGD-targeted Nexil; and, 2) determine the ability of other ovarian cancer-specific targeting ligands to enhance the efficacy of Nexil. Substantial progress has been made on both of these specific aims. This progress report covers work done during months 11 – 22 of the grant (March 2010 – February 2011).

The success of Abraxane in increasing the delivery of placitaxel (PTX) to breast cancers has sparked interest in other nanoparticle-based delivery systems that might out-perform Abraxane. CT-2103 (Xyotaq) consists of a polyglumatic acid (pGA) polymer to which PTX has been loaded to 37 %(w/w). This drug has proven safe, but has not yet been shown to have improved efficacy in phase 3 trials. We have further improved on CT-2103 by modifying the pGA backbone such that each glutamic acid in the polyer has an additional glutamic acid linked to as a side chain (pGGA). This polymer can also be loaded to a high level with PTX (35%); however, the key advantage is that the pGGA polymer loaded with PTX (pGGA-PTX) spontaneously forms a 20 nm particle in aqueous solutions and plasma. This molecule, now known as Nexil[TM], increased plasma exposure by 24-fold over that attainable with an equimolar dose of free PTX, and in the H460 lung cancer model it increased delivery of PTX ($AUC_{0-\infty}$) by 68-fold (1). We have now shown that Nexil significantly outperforms both free paclitaxel and Abraxane with respect to efficacy in multiple tumor (2).

The rationale for attaching tumor-targeting ligands to Nexil derives from the very large increase in affinity attained when multiple ligands work together to produce a Velcro-like effect. While the association rate constant increases linearly with the number of ligands, the dissociation rate falls exponentially such that the overall affinity increases markedly. The affinity can become so high that, once the dendrimer is bound, it cannot be displaced even by very high concentrations of the free ligand.

KEY RESEARCH ACCOMPLISHMENTS

Background

Despite of the improvements in the cancer treatment concerning surgical intervention, radiation and chemotherapeutic drugs, development of efficient delivery of therapeutic systems is lagged behind. This subject has been actively reviewed and it is of common agreement that nanotechnology represents an excellent opportunity to move forwards the drug delivery research (3-6).

Among the systems currently being investigated, the polymeric nanoparticles conjugates have already demonstrated promising application (4). These macromolecular prodrugs comprise

a minimun of three components: a natural or synthetic water-soluble polymeric carrier, a biodegradable polymer-drug linkage and bioactive antitumor agent. In this sense, a polyglutamate polymer loaded with paclitaxel (CT-2103, Xyotax) was described in 1998 with good *in vivo* antitumor activity (7). However, although favorable phase II clinical trial results, three randomized phase III trials in patients with non-small cell lung cancer failed to demonstrate an improvement in either progression-free or overall survival and CT-2103 has not yet received marketing approval.

Based on a polyglutamic acid polymer backbone, we have developed a new polymer where a glutamate side chain has been added to each monomer in the polymer to create polyglutamylglutamate (PGGA). When PTX is conjugated to this polymer to an extent of 35% (w/w) to create poly-(_-L-glutamylglutamine)–paclitaxel (PGGA–PTX), the tendency of the hydrophobic PTX molecules to interact with each other causes the polymer to collapse to form a nanoparticle of ~20 nm in aqueous solutions as determined by dynamic light scattering (2, 8) (Figure 1).
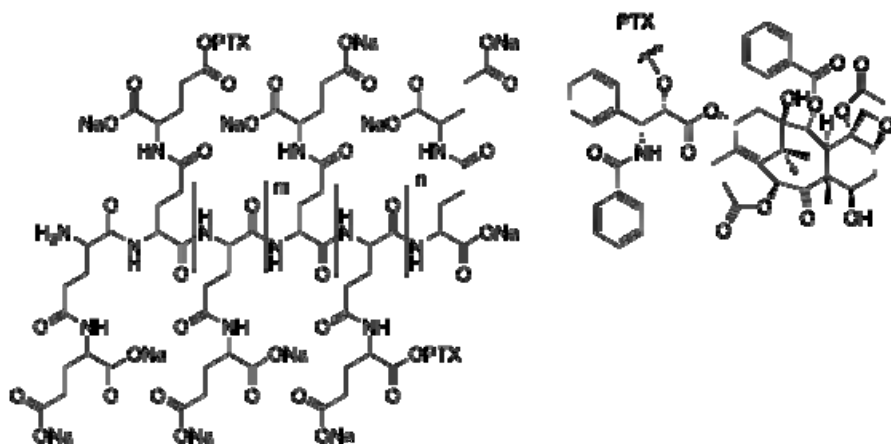

Figure 1

In our studies, this new nanoparticle, now called Nexil, has exhibited better efficacy compared to Abraxane (clinically approved paclitaxel formulation) and better pharmacokinetic parameters compared to native paclitaxel (1-2). It is very likely that the advantage of Nexil is based on its ability to passively target to the tumor due to its abnormal vasculature. Passive targeting exploits the "enhanced permeability and retention" (EPR) effect that allows nanocarriers to accumulate in the tumor. However, it is very desirable that drug delivery systems (DDS) also actively target to cancer cells. In principle, this active approach could be performed by conjugating DDS with molecules that bind to receptors specifically over-expressed on the target cells.

The **overall goal** of this research project is to further enhance the therapeutic efficacy of Nexil by targeting it to either the activated endothelial cells in tumors for anti-angiogenesis therapy, or to tumor cells themselves using peptides. The peptide chemical space is enormous and peptides are versatile in structure and conformation and highly pure peptides can be synthesized in large quantities compared to the protein ligands. Peptide ligands also generally display higher affinities for target receptors than the small molecule ligands. Although the Nexil nanoparticles are already extensively and rapidly endocytosed by tumor cells, our **hypothesis** is

that selectively can be substantially further improved using the capability of the pGGA polymer to carry a large number of tumor-specific peptides per molecule. Our present goal is to combine this novel polyglutamylglutamate nanoparticle containing paclitaxel with the known cyclic peptide (RGD) and with the tumor penetrating peptide Lyp-1. The RGD peptide exhibits good binding affinity to $\alpha_v\beta_3$ integrins which are over-expressed in tumor cells, and Lyp-1 binds to p32 found in high levels in a subset of ovarian cancer. Thus, it is expected that this new system would be able to deliver its payload (paclitaxel) to tumor cells in a very selective manner.

Specific Aim #1: RGD targeting

Synthesis of RGD-targeted Nexil

Integrins are involved in a large number of fundamental cellular processes such as cell-matrix adhesion, differentiation, proliferation, apoptosis. An important feature of these proteins, which has attracted the attention of the scientific community (9-10) is the fact that certain classes of integrins are over-expressed on tumor cells and on the endothelial cells of their capillaries. Thus, these receptors could be used as specific targets to deliver bioactive compounds against tumors in a selective way.

Based on the natural ligand of the $\alpha_v\beta_3$ integrin (vitronectin), Kessler et al, (11) have developed a cyclic peptide containing the important recognition-binding motif (arginine-glycine-aspartic acid, RGD) present on the vitronectin structure. Molecules presenting this RGD amino acid sequence and RGD mimetic can inhibit many integrins and have been considerably studied. Using the cyclic RGD peptide as tumor targeting moiety, we envisioned the possibility of synthesizing a novel conjugate based on the polyglutamylglutamate (PGGA) backbone containing paclitaxel as bioactive compound (Figure 2).
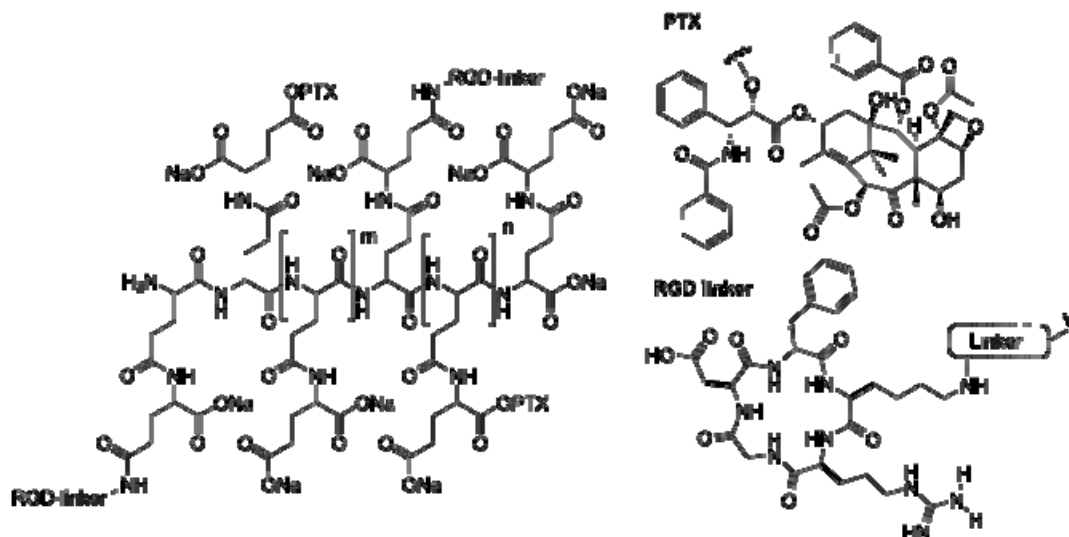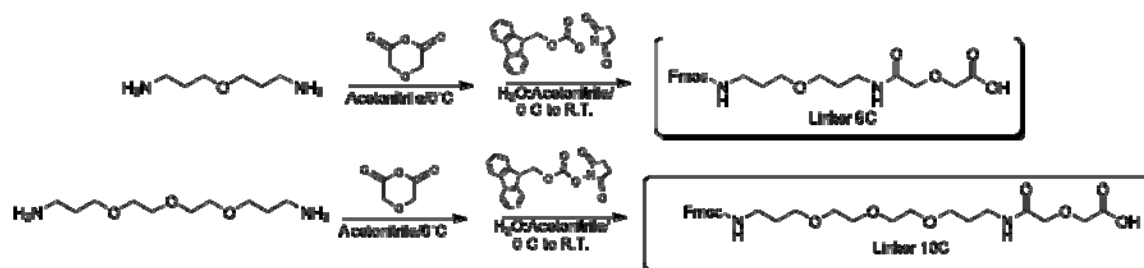

Figure 2

An important aspect to take into account in the proposed structure depicted on Figure 2 is the linker length. Precedents in the literature suggest that RGD binding activity to integrin decreases with longer linkers.(12)

We have designed and synthesized two different linkers to be used in the coupling with the RGD peptide (Scheme 1).

Scheme 1

Following the procedure described by Kessler et al,(11) the synthesis of the cyclic RGD peptide was done using Fmoc strategy on solid support (2-chlorotrityl chloride resin) and the cyclization step was performed in solution phase. The ^{1}H-NMR and the mass spectra of the peptide obtained are in agreement with the literature.

With both linkers (Linker 10C and Linker 6C) and the cyclic RGD peptide in our hands, we have proceeded to the coupling reaction between the carboxylic acid functionality present on the linkers and the lysine amino acid residue on the peptide. Using standard peptide coupling conditions and after purification in the HPLC (reverse phase), we obtained the RGD peptide coupled with the linkers in the protected form (Fmoc group). The deprotection of the Fmoc group was done using a 20% piperidine solution in DMF and the final product purified by reverse phase (HPLC) (Scheme 2).
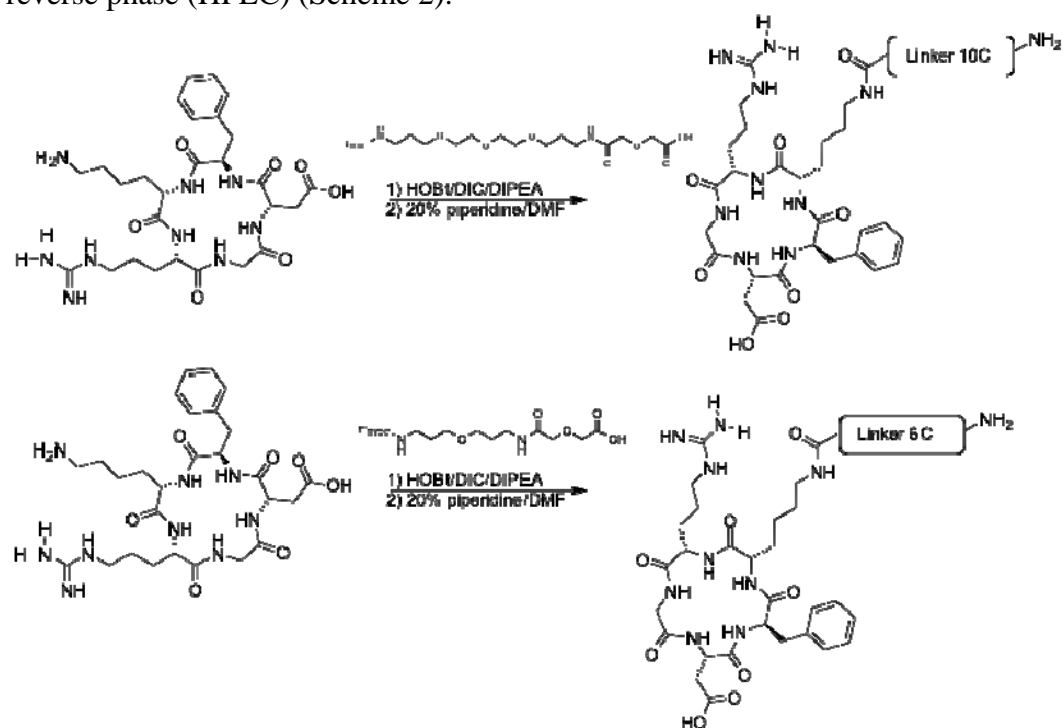
Scheme 2

In parallel, the carboxylate groups present on the polyglutamylglutamate (PGGA) backbone were activated with N-hydroxysuccinimide (NHS) for the reaction with RGD-Linker. Prior experiments in another system suggested that the optimal loading of RGD peptide is 15 units per carrier molecule. We have now synthesized a set of Nexil molecules containing 5, 15 and 30 RGD units per polyglutamylglutamate molecule (Scheme 3). These molecules were tested for their ability to bind to *in vitro* to purified $\alpha_v\beta_3$ integrin to determine their binding activity using fluorescence polarization (FPA) (13). Nexil containing an average of 15 RGD units per polymer was found to have the highest affinity and all subsequent experiments have been conducted with this molecule.
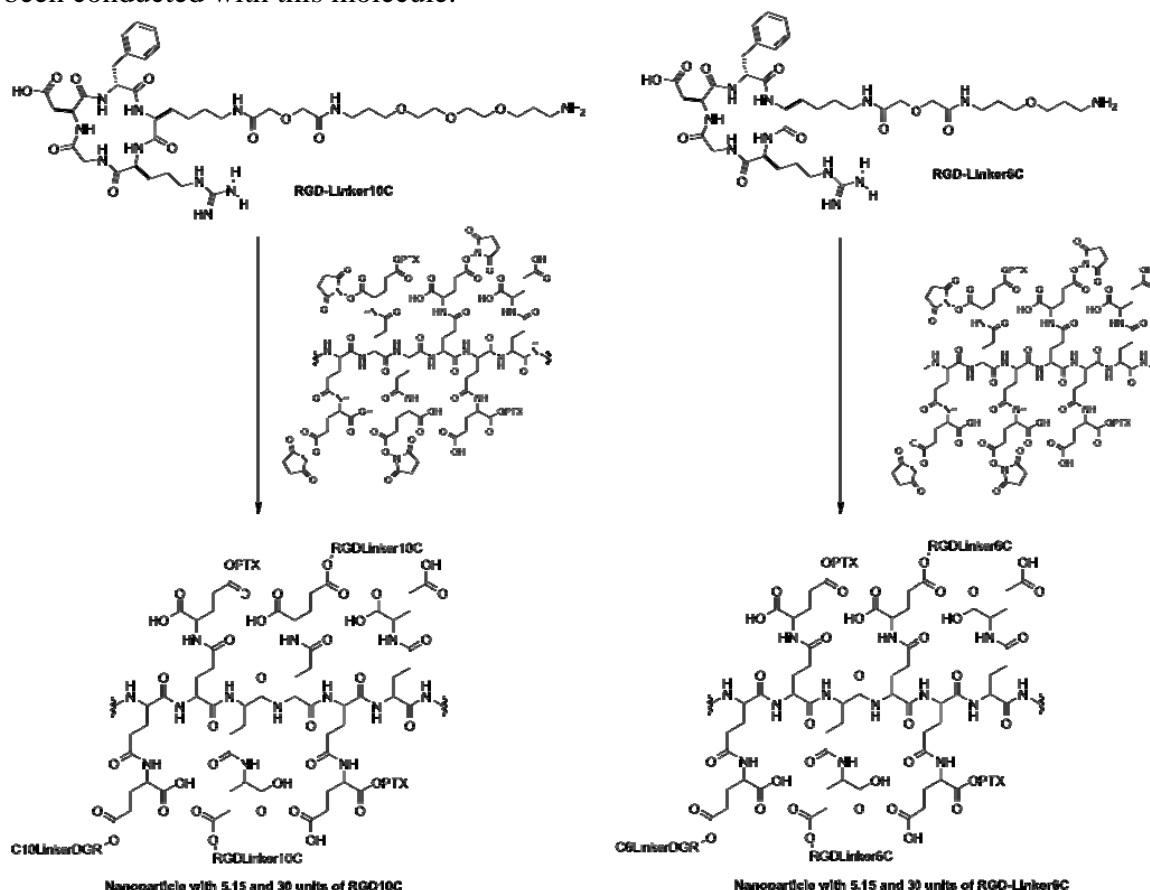
Scheme 3

In order to conduct tissue binding experiments we have also synthesized versions of Nexil containing 15 RGD per polymer into which a fluorochrome has been introduced as depicted on Scheme 3. To this aim, a part from the RGD-Linker molecules coupled to polyglutamylglutamate backbone, the known carboxyfluorescein was also attached to the polymer structure. A set of fluorescent molecules containing the RGD-Linker10C and RGD-Linker6C have now been synthesized. These fluorescent molecules will be used in a cell-based experiment with HUVEC cells(14) to determine ability of the conjugates to target tumor cells overexpressing $\alpha_v\beta_3$ integrin receptors.

To facilitate in vivo pharmacokinetic studies we have also synthesized Nexil loaded with 15 RGD per polymer using a tritiated form of paclitaxel. This has been produced in quantities in excess of 500 mg.

Correlation between the delivery by Nexil-DTPA of [111]Indium and ^{3}H-paclitaxel in human tumor xenografts

Prior to examining the pharmacokinetics of RGD-targeted Nexil we undertook a set of experiments to document that when Nexil gets to the tumor it actually delivers the paclitaxel load. Previous studies have established that Nexil out-performs Abraxane in multiple tumor models with respect to inhibition of tumor growth. We have developed a form of Nexil that is conjugated with DTPA that allows loading with ^{111}In and potentially permits ex vivo imaging of the amount of Nexil that accumulates in the tumor. In addition to its use in these experiments, the eventual goal is to use this form of Nexil to select patients for treatment with Nexil in future clinical trials. The objective of this experiment was to determine whether there is a correlation between the amount of ^{111}In and the amount of [^{3}H]-paclitaxel reaching the tumor when tested in 8 different human tumor xenografts.

Female nu/nu mice were inoculated SC with 4 x 10^6 of each of 8 different human tumor cell lines of different histologic type. Any give mouse received inoculations on each shoulder and each hip and thus carried 4 different types of tumor. At the point when the mean tumor volume for the entire population reached 400 mm^3 (6 – 7 mm diameter) each mouse received a single IV bolus injection of [^{3}H]-Nexil-DTPA-[111]In. Four hours later blood was obtained by cardiac puncture, the mice were sacrificed and the tumors dissected free of subcutaneous tissue. Each tumor was weighed and then placed in a vial and the ^{111}In dpm was counted on a gamma counter. The tumor sample was then be homogenized, mixed the scintillation fluid and the ^{3}H dpm were determined on a scintillation counter.

Twelve nu/nu mice were divided into two groups of 6 mice each. The mice in the first group were inoculated with 0.1 ml of the cell suspension in Matrigel as follows:

> Left shoulder: 2008 cells
> Right shoulder: A2780 cells
> Left hip: IGROV-1 cells
> Right hip: HEY cells

The mice in the second group were inoculated with inoculated with 0.1 ml of the cell suspension in Matrigel as follows:

> Left shoulder: KF28 cells
> Right shoulder: UCI 107 cells
> Left hip: H460 cells
> Right hip: HCT116 cells

All 12 mice were injected [111]Indium labeled [^{3}H]-Nexil-DTPA and sacrificed 4 hours after injection. The amount of indium in each tumor and plasma sample was determined using a

gamma counter and the percent of the injected dose was calculated according to the standard assumptions as to blood volume. The amount of indium in each tumor was expressed as % ID/g (percent of injection dose per gram of tumor). The data tables are included in this report as appendices. Approximate 15% of injected dose was found in the plasma in group 1 and 10 % of injected dose was detected in plasma in group 2. The large error in group 2 was because one mouse died right after anesthesia. Therefore, we had difficulty doing a cardiac puncture; some blood was obtained after opening the chest, but it was mixed with other tissues and blood and led to the high cpm count. Nevertheless this value was included that in the calculation.

Approximately 2 % of injected indium dose per gram detected in HEY, which was the highest, followed by IGROV-1, HCT116 and 2008.
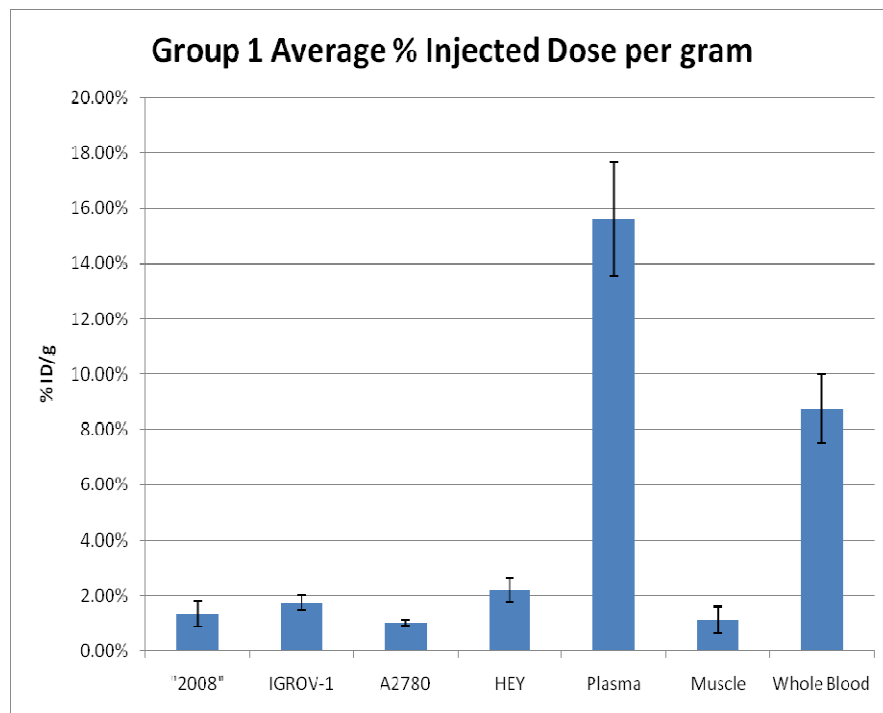
Figure 3. Average % injected dose per gram in group 1. Vertical bars, ± SD
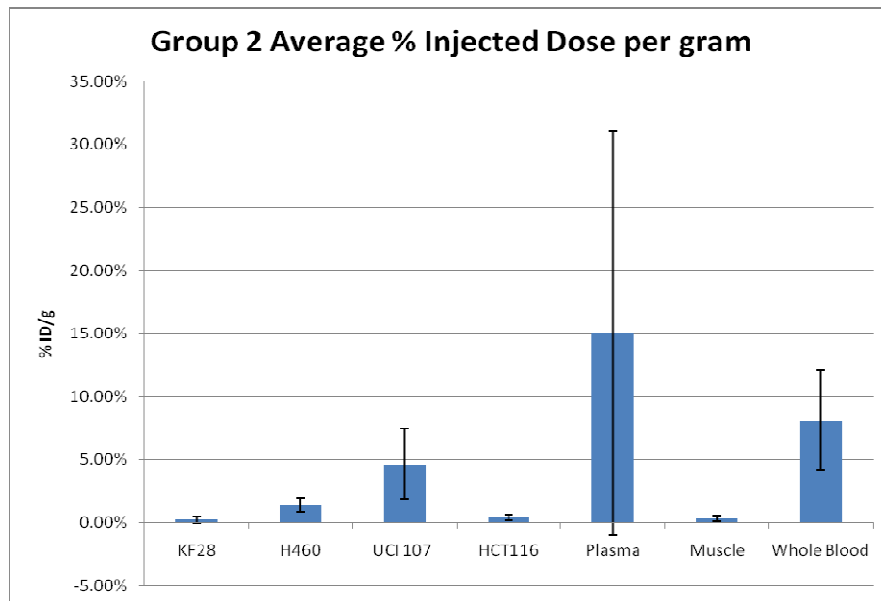
Figure 4. Average % injected dose per gram in group 2. Vertical bars, ± SD.
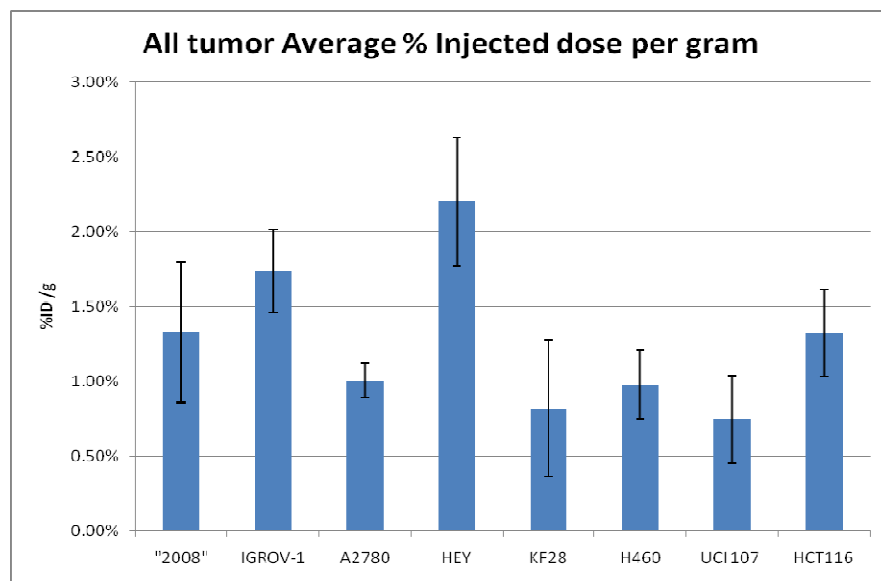
Figure 5. Average % injected dose per gram for tumors in groups 1 and 2 combined. Vertical bars, ± SD

Each tumor sample was then homogenized, mixed the scintillation fluid and the ^{3}H dpm was determined on a scintillation counter. The amount of [^{3}H]-paclitaxel in each tumor was expressed as DPM/g tumor (Figure 6). HEY also has the greatest tritium count, followed by IGROV-1, HCT116 and 2008.
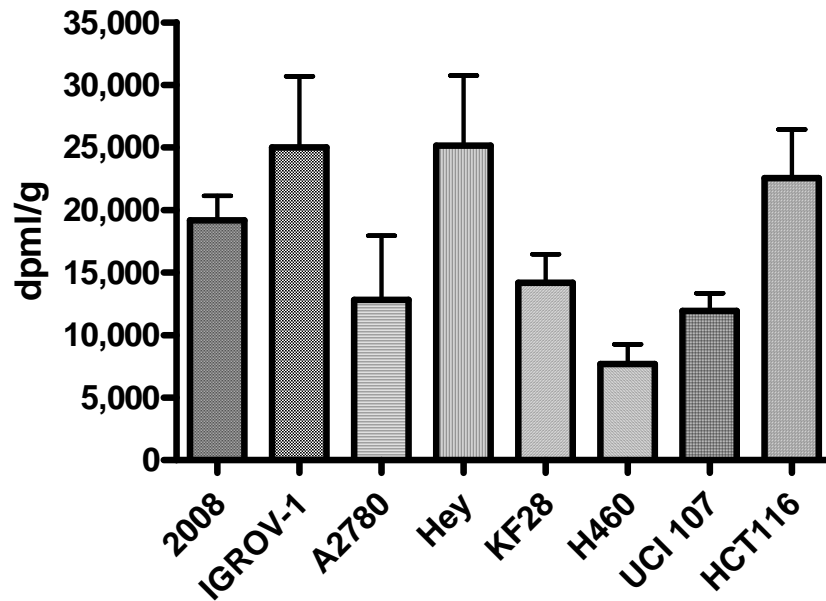
Figure 6. Average of the amount of [^{3}H]-paclitaxel/g in all tumors. Vertical bars, $\pm$ SEM

Figure 7 shows a scattergram of the ^{111}indium and ^{3}H counts per gram of weight for each of the 8 types of tumors. The correlation coefficient (r) for the delivery by Nextil-DTPA of ^{111}indium and [^{3}H]-paclitaxel across the 8 different tumor types was 0.853 (t = 4.009, p <0.01).
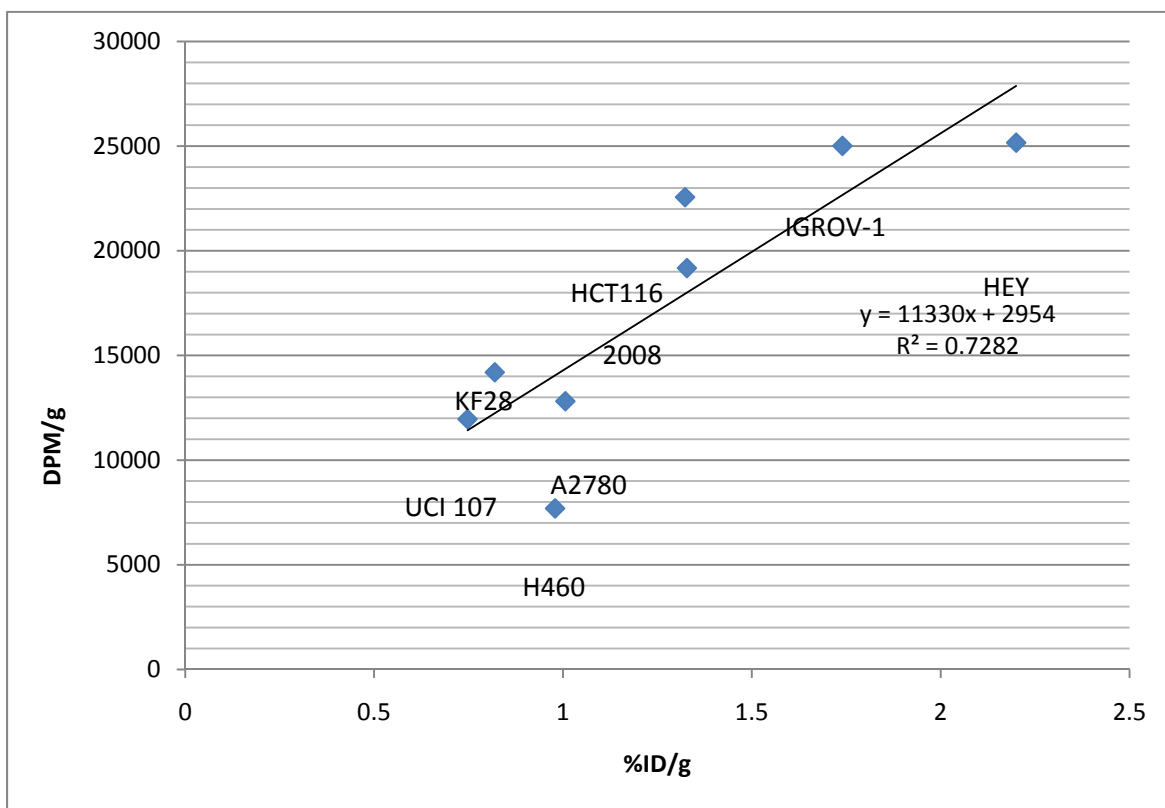
Figure 7. Correlation between the delivery by Nextil-DTPA of ^{111}indium and [^{3}H]-paclitaxel Each data point represents the mean of values for 6 mice. ^{111}Indium counts are shown on the abscissa and [^{3}H] counts on the ordinate.

The data from this experiment disclosed several important points about the behavior of Nexil. First, it is clear that there are substantial differences in the accumulation of Nexil in different types of tumors when measured at 4 hours after IV injection. Among the 8 different tumors tested in this experiment there was a >3-fold variance in the accumulation of [^{3}H]-paclitaxel and >20-fold variance for the accumulation of ^{111}indium. Assuming that there is a relationship between the amount of Nexil that accumulates in a tumor and the probability of response, there is thus a clear opportunity to use trace injections of Nexil-DTPA as a tool with which to select patients for treatment with Nexil.

A second important point is that there was a very good correlation between the accumulation of ^{111}indium and [^{3}H] paclitaxel across the panel of the 8 different tumors used in this study. The correlation coefficient was 0.853. Thus, we can conclude that when both radioactive labels are on the same polymer, when a high accumulation of the ^{111}indium is accompanied by a high accumulation of paclitaxel. This observation suggests that the accumulation of Nexil-DTPA, which can be quantified in patients using a gamma camera, is a valid surrogate measure of the ability of Nexil to deliver paclitaxel to the tumor.

In the current experiment the [³H]-paclitaxel and the ¹¹¹indum labels were on the same Nexil molecules. To further validate Nexil-DTPA as a tool with which to select patients for treatment with Nexil in clinical trials, it would be helpful to repeat this experiment injecting a mixture Nexil-DTPA loaded with ¹¹¹indium and [³H]-paclitaxel-Nexil with the two labels on separate Nexil molecules.

The next most important step in the validation of Nexil-DTPA as a patient selection tool would be to prove that tumors that take up a lot of Nexil-DTPA are more sensitive to the therapeutic effect of Nexil than tumors that take up only a small amount. It would be informative to compare the therapeutic efficacy of Nexil against the HEY versus the UCI107 tumors which represent the extremes of ¹¹¹indium uptake.

Comparison of the tumor and plasma pharmacokinetics of Nexil and RGD-conjugated Nexil

Previous studies have established that Nexil out-performs Abraxane in multiple tumor models with respect to inhibition of tumor growth. Nexil has now been conjugated with the RGD peptide (15 RGD per polymer) in an attempt to target it to tumor and tumor endothelial cells expressing activated integrins. The goal of this experiment was to determine whether RGD-conjugated Nexil accumulates to higher levels in human lung carcinoma H460 xenografts than unconjugated Nexil.

A total of 48 female nu/nu mice were inoculated SC with 4×10^6 H460 lung cancer cells at 4 sites (left and right shoulder and left and right hip). At the point when the mean tumor volume for the entire population reached 400 mm³ (6 – 7 mm diameter) the mice were randomly allocated into two groups of 24. Mice in group 1 received a single IV bolus injection of [³H]PTX-Nexil and mice in group 2 received a single IV bolus injection of [³H]PTX-Nexil-RGD. At 10 minutes and 1, 4, 12, 24, 48, 96 and 192 h after injection 3 mice from each group were sacrificed. Blood was obtained at the time of sacrifice, and after sacrifice the tumors were dissected free of subcutaneous tissue. Each tumor was weighed, homogenized, mixed with scintillation fluid and the ³H dpm were determined on a scintillation counter. A graph was constructed of plasma and tumor concentrations of total [³H]PTX as a function of time.

Plasma concentration of [³H]PTX

Forty-eight mice were randomly allocated into 2 groups. Mice in group 1 received a single IV bolus injection of [³H]PTX-Nexil and mice in group 2 received a single IV bolus injection of [³H]PTX-Nexil-RGD. Blood samples were collected at 0.16, 1, 4, 12, 24, 48, 96, 192 hours after injection. One hundred µL of plasma was collected, transferred to scintillation vials and counted on a scintillation counter. The amount of [³H]-paclitaxel in each plasma sample was expressed as DPM/g plasma. Over the first 4 h there was a trend toward a lower plasma tritium concentration in the mice injected with [³H]PTX-Nexil-RGD. There was a statistically significant difference in the plasma concentrations at 0.16 (p =0.048) and 4 h (p = 0.0252) using two-tailed paired *t* test for unpaired samples in the GraphPad Prism4 software. The area under the concentration-times-time curve for [³H]PTX-Nexil-RGD was 56.2% of that for [³H]PTX-Nexil.
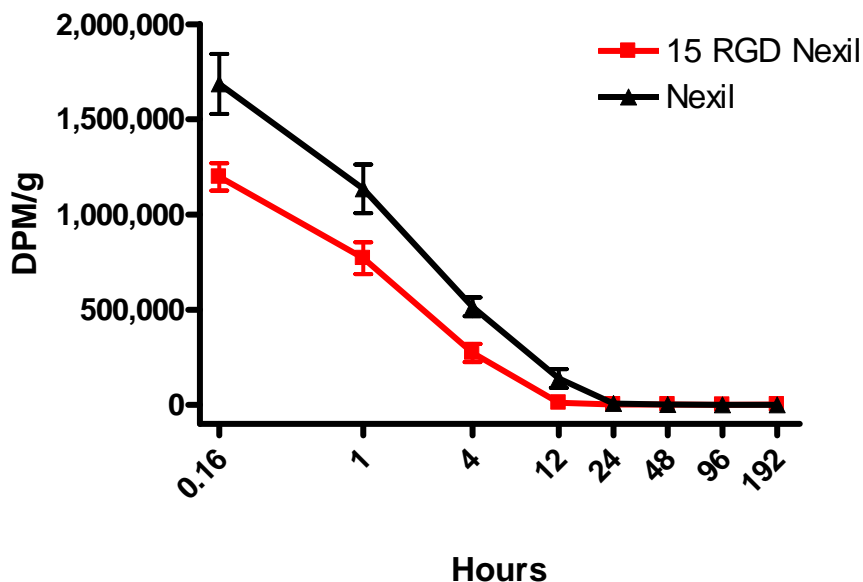
Figure 8. [³H]PTX levels in plasma. Each point represents the mean of three plasma samples from three mice. ■: Mice treated with 15 RGD Nexil; ▲: Mice treated with unconjugated Nexil; Points: Mean ± SEM.

Tumor concentration of [³H]PTX

Tumors were collected at 0.16, 1, 4, 12, 24, 48, 96, 192 hours after injection from the same 48 mice in which plasma had been collected. Samples were homogenated and counted on a scintillation counter. The amount of [³H]-paclitaxel in each tumor sample was expressed as DPM/g tumor. Among 4 tumors from each animal, the most obvious outlier was removed from each group. The average of [³H]-paclitaxel from the remaining three tumors in any given mouse was calculated and plotted in Figure 9 as a function of time and treatment. There was a statistically significantly higher tumor content of [³H]PTX-Nexil rather than [³H]PTX-Nexil-RGD at 24 ($p = 0.02$) and 96 h ($p = 0.02$). The area under the content-times-time curve for [³H]PTX-Nexil-RGD was 60.4 % of that for [³H]PTX-Nexil.

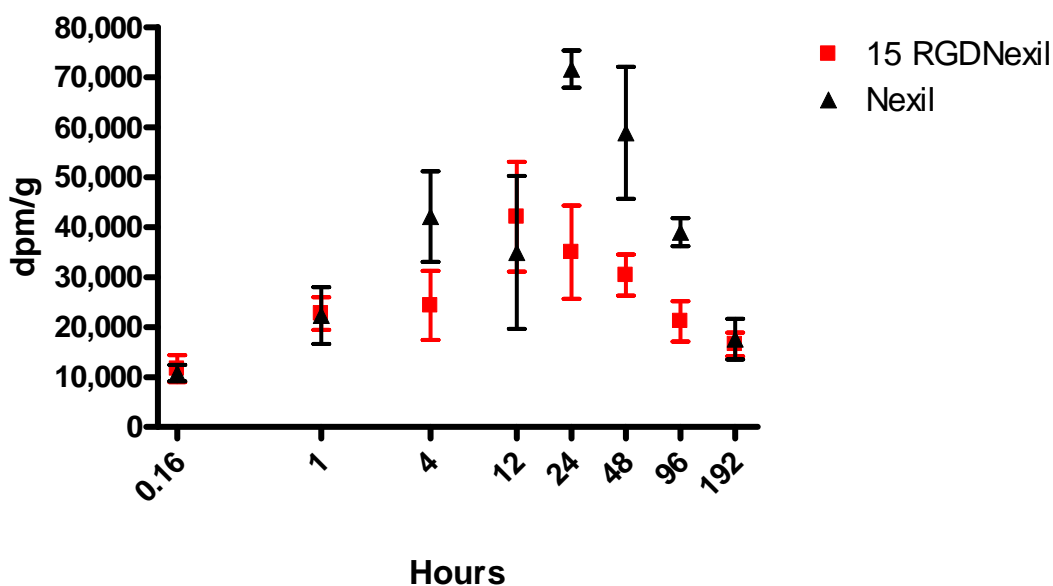*Figure 9. [³H]PTX levels in tumors. Each point represents the mean of 3 mice. ■: Mice treated with 15 RGD Nexil; ▲: Mice treated with unconjugated Nexil; Points: Mean ± SEM.*

The RGD peptide has good binding affinity to activated $\alpha_v\beta_3$ integrin receptors which are commonly expressed on the endothelial cells of tumors and often on the tumor cells themselves. It was expected that addition of 15 cyclic RRD peptides per Nexil polymer would increase the delivery of paclitaxel to tumors in a selective manner. However, the RGD-conjugated Nexil did not perform any better than the Nexil itself. RGD-Nexil reached a lower maximum plasma concentration than Nexil when measured at 0.16 h after injection and this trend toward lower plasma concentration continued for the first 4 hours after injection. Interestingly, there was no corresponding difference in the tumor content of RGD-Nexil and Nexil during the same period. Using just the exponential phase of the plasma decay curve over the first 12 h, the half-life of the RGD-Nexil was estimated to be 1.6 h and that for Nexil 1.9 h.

The tumor content of tritiated paclitaxel reached a peak at 24 h following injection of Nexil and at 12 h following injection of RGD-Nexil. The maximum peak tumor content for RGD-Nexil (at 12 h) was only 59.2 % of that for Nexil at 24 h. Following the peak content, the washout of RGD-Nexil and Nexil was similar. Non-linear regression estimated the washout half-life to be 78 h for RGD-Nexil and 82 h for Nexil.

To provide further insight into the effect of adding 15 RGD per Nexil to the performance of Nexil, the cytotoxicity of Nexil was compared to that of RDG-Nexil in two cell line model systems. The first consisted of an isogenic pair of human melanoma cell lines one of which expresses the αV integrin (M21) and the other of which does not (M21L). The second consisted of the human lung carcinoma cell line H460. As shown in Figure 10, there was no significant difference in the cytotoxicity of RGD-Nexil against either the αV-expressing or αV non-expressing melanoma cell line, suggesting that the RGD loaded onto Nexil was not capable of engaging integrin receptors in a manner that resulted in greater paclitaxel delivery into the cells in tissue culture. The IC_{50} for the M21 cells was 0.53 µM and for the M21L line it was 0.74 µM.
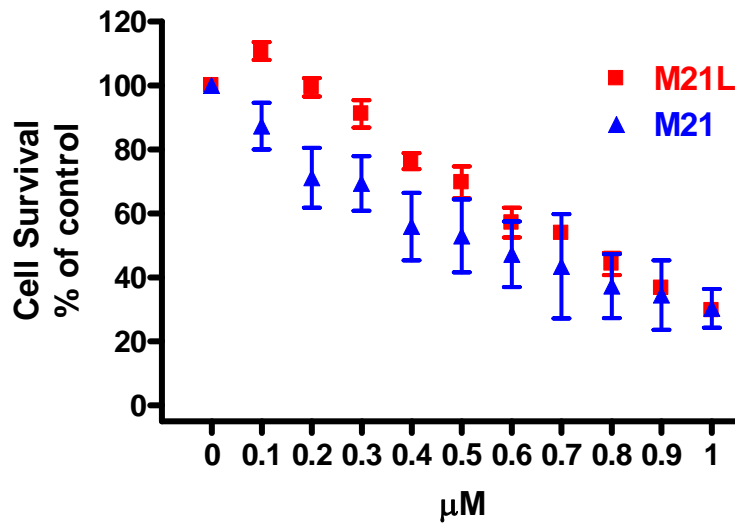
Figure 10. The CCK8 assay was used to assess the cytotoxicity of RGD-Nexil against an isogenic paired of human melanoma cells one of which expressed the αV integrin (M21) and the other of which did not (M21L). Both types of cell were exposed to drug for 72 hours. OD 450 nm was measured using a microplate reader. Points, mean of at least three experiments each performed with triplicate cultures.

As shown in Figure 11, RGD-Nexil was somewhat less toxic to H460 cells in vitro than Nexil. The IC_{50} value for Nexil was 0.6 µM; that for RGD-Nexil was clearly greater but could not be calculated from the data available.
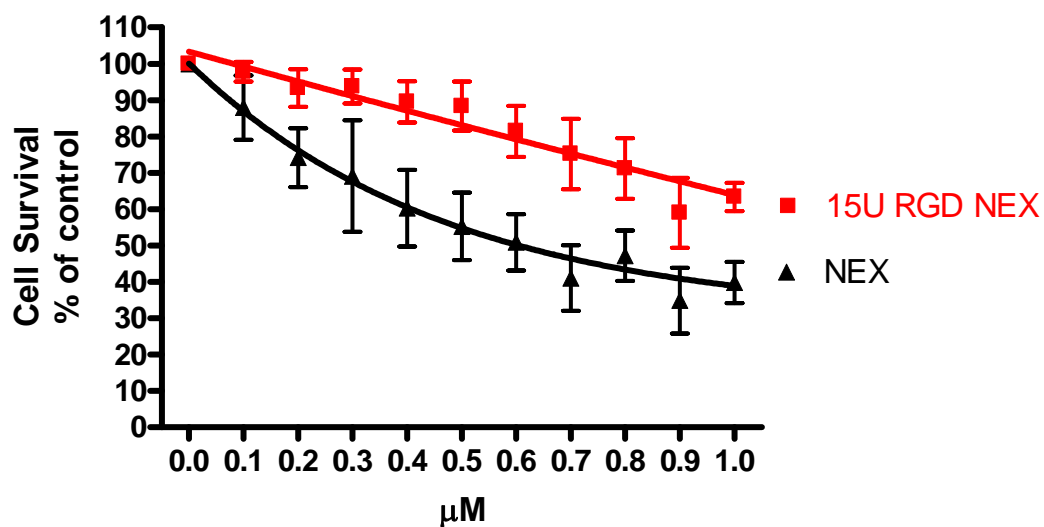
Figure 11. The CCK8 assay was used to measure assess the cytotoxicity of Nexil and RGD-Nexil against the H460 lung cancer cell line in a 72 h assay. OD 450 nm was measured using a microplate reader. Points, mean of at least three experiments; each done in triplicate)

Specific Aim #2: Lyp-1 targeting

Lyp-1 is a member of a novel class of peptides that can markedly and selectively increase the penetration of drugs into tumor nodules (15). Lyp-1 was originally isolated from a phage display library on the basis of its ability to direct phage to MDA-MB-435 tumor xenografts. (16) This compound has proved to bind specifically to tumor lymphatic and tumor cells, leading to cell death by apoptosis and inhibiting tumor growth in mice bearing breast cancer xenografts. The attractive feature of Lyp-1 is the ability of this compound to be internalized by tumor cells, which can be explored for drug delivery purposes.(17) Recently, Ruoslahti et al have suggested the internalization mechanism of Lyp-1 is through the known receptor p32 that is present and overexpressed at the tumor cell surface (18).

Our goal in this Specific Aim is to synthesize Lyp-1 and use it to enhance targeting of Nexil to tumors (Figure 1).

Lyp-1 peptide
(Cys-Gly-Asn-Lys-Arg-Thr-Arg-Gly-Cys)

Figure 1

Precedents in the literature support our hypothesis that the efficacy of Nexil can be further improved using Lyp-1 as targeting moiety. Nanoparticles of iron oxide,(19) albumin-based (20) and even baculovirus combined with Lyp-1 have demonstrated excellent results for binding tumor cells (21).

Synthesis of Linkers for RGD and Lyp-1 attachment to Nexil Backbone RGD linker 6C (short linker)

        The starting material (4,7,10-trioxa-1,13-tridecanediamine) was dissolved in dry acetonitrile (130mL) under nitrogen atmosphere at room temperature. This solution was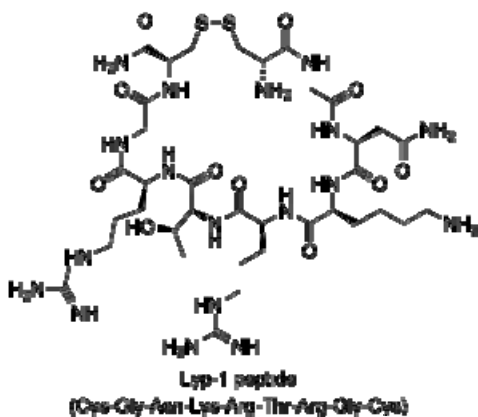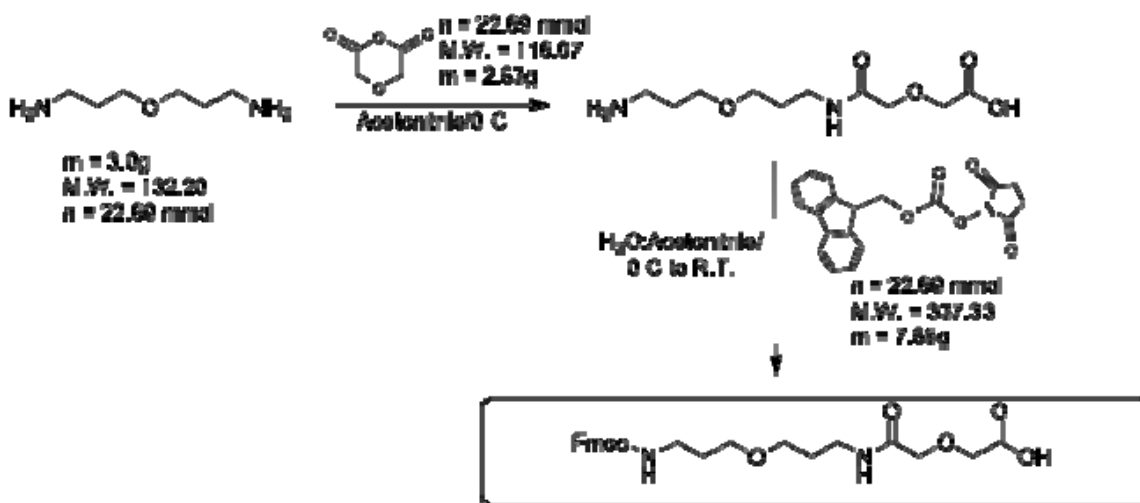 cooled to 0˚C using a Dewar containing ice. The amount of diglycolyl anhydride calculated was also dissolved in acetonitrile (20mL) at room temperature and it was added slowly to the starting material solution using a syringe pump system. The addition was completed after 30 minutes and there was a formation of a "milky" solution, which was stirred at room temperature for another 2 hours. To this reaction it was added 150 ml of water and the reaction became clear and it was cooled at 0˚C with an ice bath. Then, 3.5mL of DIPEA (diisopropylethylamine) was added to the mixture and the reaction was stirred for 5-10 min. The Fmoc-OSu calculated was dissolved previously in a solvent mixture 1:1 of acetonitrile:CH_2Cl_2 (30mL) and it was added to the reaction and the ice bath was removed . Another 2.0mL of DIPEA was added to keep the reaction pH basic and the reaction was stirred overnight. The reaction was quenched evaporating part of the solvent in the rotavaporator and the remaining solvent was acidified with HCl 3M solution until pH 2-3 and the reaction was extracted several times with ethyl acetate. The organic phase was dried over $NaSO_4$ and the solvent removed in the rotavaporator. The crude obtained was purified in a Combiflash system (regular silica gel) with a solvent gradient of MeOH (containing 10% of water)/CH_2Cl_2. The overall yield (after 2 steps) was 68%.

RGD linker 10C (long linker)

        Exactly same procedure as described for the short version of RGD-linker was used for the synthesis of this linker.

After purification the overall yield (after 2 steps) was 42%.

Lyp-1 linker

Due to an important lysine residue in the Lyp-1 structure, the linker designed for the RGD peptide is incompatible for the conjugation with the Nexil backbone. The Lyp-1 peptide requires a linker with an orthogonal reactivity towards the different amino acids residues present in the peptide. The maleimide group and/or azido group meet this condition for the conjugate synthesis. The maleimide group presents excellent selectivity for sulfhydryl functionalities present in the cysteine or cysteine derivatives. On the other hand, the azido group can react with alkyne functionality (click chemistry) being widely used for conjugation purpose by several groups.

The synthesis of the linker for the Nexil-Lyp1 conjugate was successfully accomplished as depicted in the Scheme 1

Scheme 1

The commercially available pentaethylene glycol was dissolved in dry DCM at room temperature and under nitrogen atmosphere. To this solution it was added the glycine and the DMAP. The solution was cooled at 0°C and the DIC was added to the reaction. After 10 minutes the reaction was allowed to warm up until room temperature and the reaction was stirred for another 4 hours. After this time, the solvent was removed in the rotavaporator and the residue purified in chromatographic column using a gradient mixture of DCM:MeOH (2%, 5% and 10% MeOH). The mono glycine glycol was then dissolved in THF (in a microwave tube) at room temperature and to this solution was added the Ph_3P, maleimide and DEAD. The reaction was then heated using the microwave (85°C) during 30 minutes. After this time the reaction was transferred to a round bottom flask and the solvent removed in the rotavopator and residue was

purified in chromatographic column using a solvent gradient DCM:EtOAc (20%, 40% and 80% EtOAc).

The final compound was obtained after deprotection of the Boc group using a solution of TFA (30%) in dichloromethane at room temperature. The solvent was evaporated in the rotavaporator and the crude obtained was dissolved in water and washed 2 times with a solvent mixture of hexane(70%)/ethyl acetate (30%). The aqueous phase was lyophilized providing the desired compound with excellent yield (94%).

RGD synthesis

After swelling the Chlorotrityl resin with anhydrous dichloromethane, the synthesis of the RGD peptide has followed the amino acid sequence depicted in scheme 2.
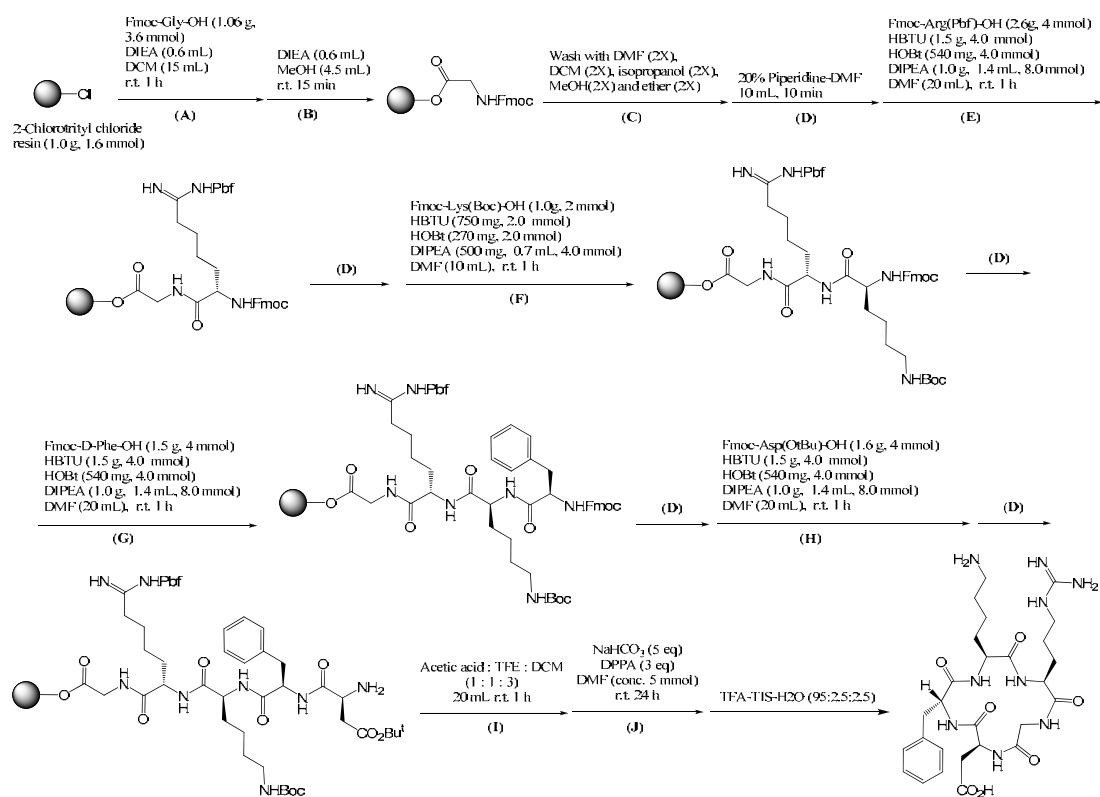
Scheme 2

After releasing the peptide sequence from the solid support (step I), the linear peptide was purified using reverse phase preparative HPLC. As eluent it was used a gradient of acetonitrile (with 0.1% of TFA) water (with 0.1% TFA) with a solvent flow of 8ml/min. The cyclization step was performed as described previously in the literature (11). In a round bottom flask the linear peptide (1.2g, 1.16mmol) was dissolved at room temperature in dry DMF (232mL) under N_2 atmosphere. To this solution, it was added $NaHCO_3$ (0.487g, 5.8mmol) and the mixture was stirred during 20min at room temperature. After this time, diphenyl phosphoryl azide (DPPA, 0.958g, 3.48mmol) was added and reaction was stirred overnight at room temperature. The reaction was monitored using TLC (10% MeOH: 90% CH_2Cl_2) and it is possible to observe that the starting material had been completely consumed. The product is less

polar than the starting material (Rf=0.6 using the eluent mentioned before). The reaction was quenched with H_2O followed by extraction with EtOAc (6 times). The organic phase was dried over Na_2SO_4 and filtered. After removing the solvent in the rotavaporator, the crude obtained was purified in chromatographic column (CombiFlash system) using a gradient of MeOH in CH_2Cl_2. The LC/MS has indicated the correct mass of the desired compound $[M+1]^+ = 1013$.

The cyclic peptide was then deprotected using the conditions shown in the scheme 1. The starting material was suspended in CH_2Cl_2 (20mL) and cooled at 0°C with an ice bath. The mixture of TFA:TIPS:H_2O (95%:2.5%:2.5%, 20mL) was added slowly and the solution has turned clear after the complete addition of solvent mixture. The ice bath was removed and reaction was stirred for 3 hours at room temperature. The reaction was monitored using TLC and after its completion the solvent was removed in the rotavaporator. The crude obtained was washed several times with Et_2O (at least 6 times) and residue obtained was dissolved in a mixture of MeOH:H_2O (85%MeOH:15%H_2O)(22) and purified in the reverse phase preparative HPLC (acetonitrile with 0.1% of TFA and water with 0.1% TFA, with a solvent flow of 8ml/min). The final compound was characterized by H^1NMR (Figure 1) and LC/MS ($[M+1]$ = 604) and compared with the compound published in the reference 1 (see supporting information)(11). The chemical yield after 2 steps (cyclization and deprotection) was 71%.


Lyp-1

Synthesis

The synthesis of Lyp-1 peptide was performed following the amino acid sequence and reaction conditions depicted in the Scheme 3. The Rink amide resin was swelled with anhydrous dichloromethane during one hour. After this time the swollen resin was washed with DMF and Fmoc protecting group present in the resin was removed under the typical condition for Fmoc deprotection (20% piperidine in DMF). With the amino group in the exposed, the peptide synthesis proceeded using the same conditions as described for the RGD peptide. Once the whole amino acid sequence was completely attached to the solid support, the last cysteine residue was deprotected and reacted with linker under the coupling conditions. The resin containing the peptide sequence and the linker was treated with a mixture of 2-mercaptoethanol/DMF in 1:1 proportion during 5 hours at room temperature. (23) The resin was washed with DMF. DCM**,** DMF, ethanol and DCM. The free thiol group in the second cysteine residue was re-protected with 5 fold excess of 2,2'-dithiobis(5-nitropyridine)(DTNP). In this step the DTNP was first dissolved in anhydrous DCM at room temperature in a different flask and this solution was added to the flask containing the resin. The reaction was agitated during 3 hours at room temperature and the excess of DTNP was removed by filtration and washing the resin several times with DCM. (24)

The disulfide bond formation between the cysteine residues present in the peptide was performed adding a 1%TFA in DCM solution at room temperature and agitating the resin during 1 hour (see ref.4) and washed with DCM. Finally, the protecting group present on the linker (Trityl) and the peptide was removed from the solid support in one step, stirring the resin with a mixture of TFA/TIS/H_2O (95/2.5/2.5). For ensuring the complete recovering of the peptide the resin was washed twice with TFA. The TFA was removed in the rotavaporator and residue was suspended in water and the aqueous phase was extracted several times with cold ether. This extraction was done for removing the side products of the deprotection side products. The aqueous phase was lyophilized and the crude peptide was purified in the preparative HPLC, using acetonitrile (0.1% TFA) and water (0.1%TFA) as eluent and molecular weight of the peptide was confirmed by MALDI/TOF spectrometry.
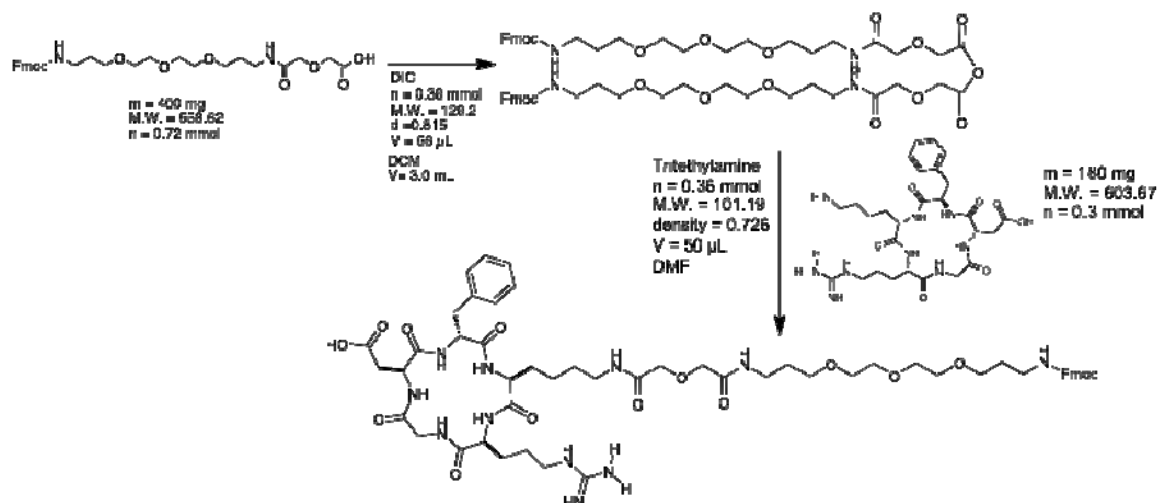
Scheme 3

RGD peptide coupling with linker

Before attaching the RGD peptide to the Nexil backbone, the peptide was reacted with the linkers described previously using two different approaches as shown in the Scheme 4.

Approach 1
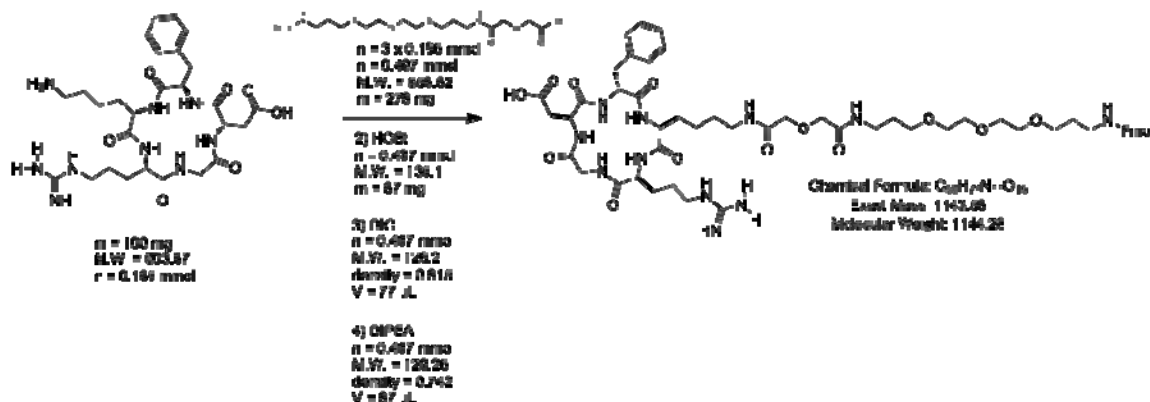
Approach 2

Scheme 4

Although both approaches have provided desired compound, the second approach has leaded to the final product with better yield and cleaner. Moreover, the second approach has the advantage to recover the linker due to the nature of the anhydride reaction. Scheme 5

The reaction between the linker anhydride and the RGD peptide is a two steps process. In the first step the linker reacts with itself to generate the anhydride, in this case the linker was dissolved in anhydrous DCM at 0˚C (ice bath) followed by the addition of the coupling reagent DIC. The ice bath was removed and the reaction was allowed to warm up to room temperature. After 1 hour, the DCM was removed in the rotavaporator and the residue obtained was dissolved in anhydrous DMF. In another round bottom flask the RGD peptide was dissolved in anhydrous

DMF and to this solution triethylamine was added and the mixture was stirred during 10 minutes. The linker anhydride was then added to the peptide solution solution and the reaction was stirred during 2 hours. The reaction was quenched adding HCl solution 1M until pH 3, followed by addition of water and ethyl acetate. The two phases were separated and the aqueous phase was extracted another 3 times with ethyl acetate. The organic phase was put it together (containing the linker) and the aqueous phase was evaporated in the rotavaporator affording the peptide coupled with the linker.
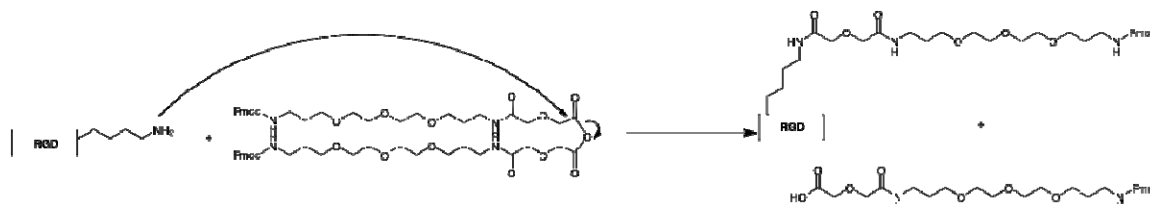
Scheme 5

The crude of the reaction between the RGD peptide and linker was then dissolved in DMF and to this mixture a solution containing 20% piperidine in DMF for removing the Fmoc group present in the linker. After 1 hour the solvent present in the reaction was removed in the rotavaporator and residue obtained was acidified (HCl solution 1M) followed by addition of water and ethyl acetate. The aqueous phase was extracted another 3 times with ethyl acetate and then lyophilized. The crude peptide containing linker was purified in the preparative HPLC affording the desired compound to react with the activated Nexil backbone.

Nexil backcbone activation
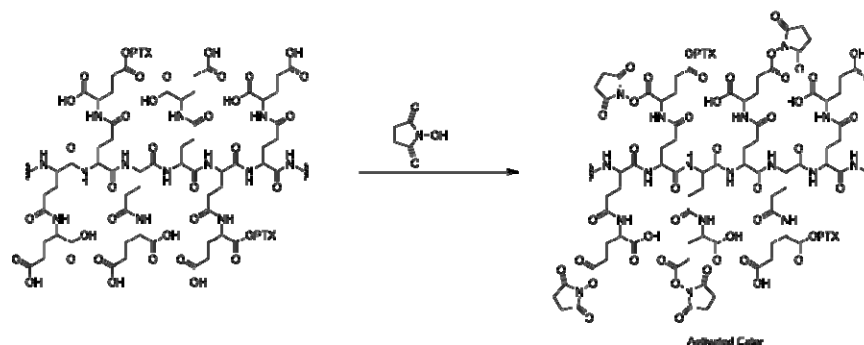
The activation of the polymer backbone of Nexil is a common precursor for Lyp-1 and RGD conjugates. However, the Lyp-1-Nexil conjugate requires and extra step until the final product and will be discussed later in this report. The amount of the targeting moiety (RGD and/or Lyp-1) per polymer backbone was considered in three different proportions (30, 15 and 5 units). These 3 formulations can provide valuable information about the influence of the multivalence effect of the tumor homing peptides in our molecules. As the highest proportion for both peptide is 30 units per polymer backbone, the number of carboxylate groups to activate is calculated with a slightly excess. Thus, the reaction with HO-Su is calculated for 35 units of carboxylate group. The coupling reagent for this reaction is EDC and follows equimolecular relation with HO-Su. In a round bottom flask 300mg of Nexil in acidic form was dissolved in dry

DMSO at room temperature and under nitrogen atmosphere. The carboxylate number of mol in 100 mg of Nexil is calculated as follows:

In 100mg of Nexil we have 63mg of polymer backbone, so the number of mol will be
n = 63/34K
n = 1.85×10^{-6}
 So in 300mg of Nexil the carboxylate number of mol is:
n = 1.85×10^{-6} x 3
n = 5.55×10^{-6}

As we want to activate 35 units of carboxylate
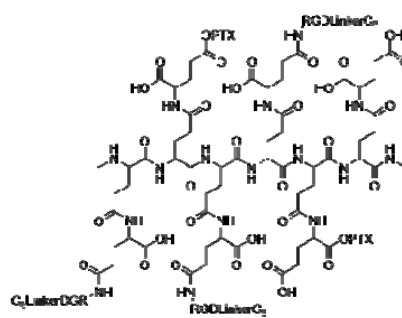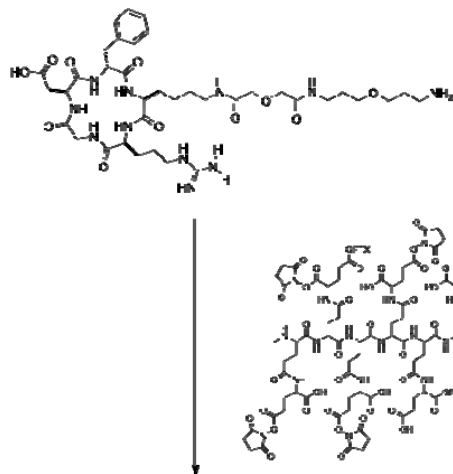n = 1.85×10^{-6} x 35
n = 1.94×10^{-4}

To the Nexil solution it was added HO-Su (MW= 115.09g/mol, m = 22mg) followed by EDC (MW= 191.70 g/mol, m = 37mg) previously dissolved in DMSO and reaction was stirred during 20 hours at room temperature. The reaction was quenched adding EtOH and Et_2O anhydrous and the precipitated and the organic solution was transferred to a centrifuge tube. The tube was centrifuged at 3600 rpm and the solvent was discarded. The precipitated was again suspended in anhydrous EtOH and centrifuged once again. The precipitated was then transferred to a round bottom flask and was dry in the vacuum pump overnight. Even after this time the NMR spectrum presents EtOH resonance peaks. The difference between the Nexil and activated Nexil NMR spectra is very subtle, where the activated Nexil NMR spectrum presents an extra shoulder in the region of 2.7-2.8 ppm.

Synthesis of RGD-Nexil conjugates

With all the starting material (RGD-linker and activated Nexil) in hand, the synthesis of the conjugates is simple. In the RGD-Nexil case the excess of carboxylate groups activated can be easily hydrolyzed in the purification step (dialysis). Thus, for the formulation where we have 5 and 15 units of the RGD peptide the excess of carboxylate group will be transformed into respective carboxylic acid.
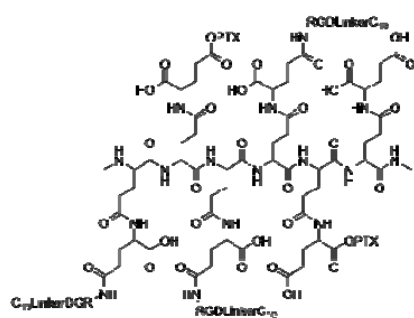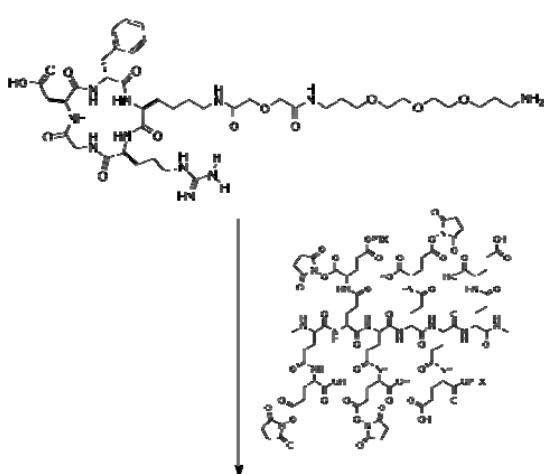
Synthesis of Axol with 6,15 and 30 units of RGD   Synthesis of Nexo with 5,15 and 30 units of RGD

For each 100mg of Nexil, 63mg is PGGA, so the number of mol of GGA (glutamyl glutamic acid units) is 63mg/34000 [259.24 (GGA unit M.W.) x 131 (GGA units per polymer backbone)], which is $1{,}85.10^{-6}$. For Nexil-RGD 30 units the mass of RGD-linker 10C needed in the synthesis is n = 30. $1{,}85.10^{-6} = 5{,}55.10^{-5}$. 922.04 = **51 mg.** Same calculation base was used for 15 and 5 RGD units for polymer backbone.

In a round bottom flask the amount of activated Nexil (100 mg) was dissolved in DMF (5mL) at room temperature, to this solution the peptide (51mg) was added followed by triethylamine (10μL) and reaction was stirred overnight. The reaction was quenched adding a solution of sodium bicarbonate and the reaction was transferred to a dialysis bag (10.000 MW) and stirred overnight. The compound was lyophilized providing the desired compound for *in vitro* and *in vivo* studies.

Calculation of RGD percentage in the conjugate

For calculating the RGD percentage in the conjugate we have to estimate theoretically the molecular weight of the whole molecule (Nexil + RGD-linker molecule). Based on this number, it is possible to calculate the percentage of RGD-linker molecule in the three different formulations. Thus, the conjugate molecular weight will follow the equation: M.W. = 51554 (Nexil) + n x 922.04 (RGD-linker10C) – n x 18 (where n is the number of RGD units por unit of Nexil backbone, 922.04 is the M.W. of RGD with the linker 10C attached to it and 18 is water molecular weight).

Chemical Formula: C₄₁H₆₆N₁₀O₁₃
Molecular Weight: 822.04

RGD-Linker C10 (compound available)
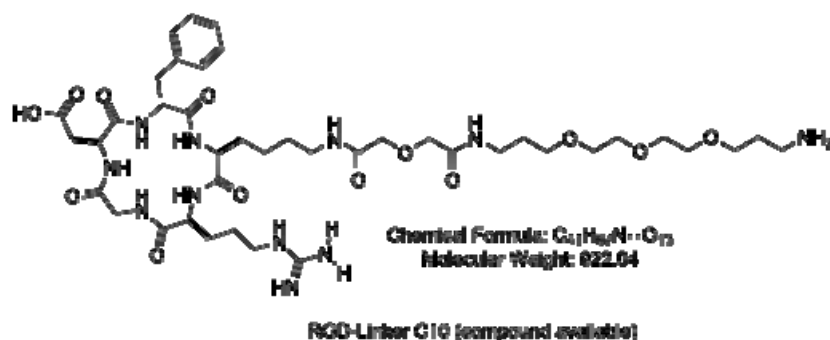
- Conjugate Nexil-RGD (30 units)
M.W. = 51554 + 27661 -540 = 78675
RGD linker represents a 35.1% of the conjugate molecular weight.

- Conjugate Nexil-RGD (15 units)
M.W. = 51554 + 13831 – 270 = 65115
RGD linker represents a 21.1% of the conjugate molecular weight.

- Conjugate Nexil-RGD (5 units)
M.W. = 51554 + 4610 -90 = 56074
RGD linker represents an 8.2% of the conjugate molecular weight.

Nexil-Lyp-1 linker synthesis

As mentioned previously, the Lyp-1 structure presents an important lysine residue that hampers the synthesis of the conjugate using the same protocol described for RGD peptide. Moreover, the required maleimide group for thiol conjugation is very sensitive under basic conditions, which also represent an extra challenge due to the incompatibility for purifying by dialysis.

The synthesis of Lyp-1 conjugate has to be performed in two steps process. The first step is the synthesis the Nexil-Lyp-1 linker using the activated Nexil and the Lyp-1 linker. The second step is the conjugation between the Lyp-1 peptide and Nexil-Lyp-1 linker.

First Step- Nexil Lyp-1 linker coupling

Activated Ester → Nexil-Lyp-1 linker
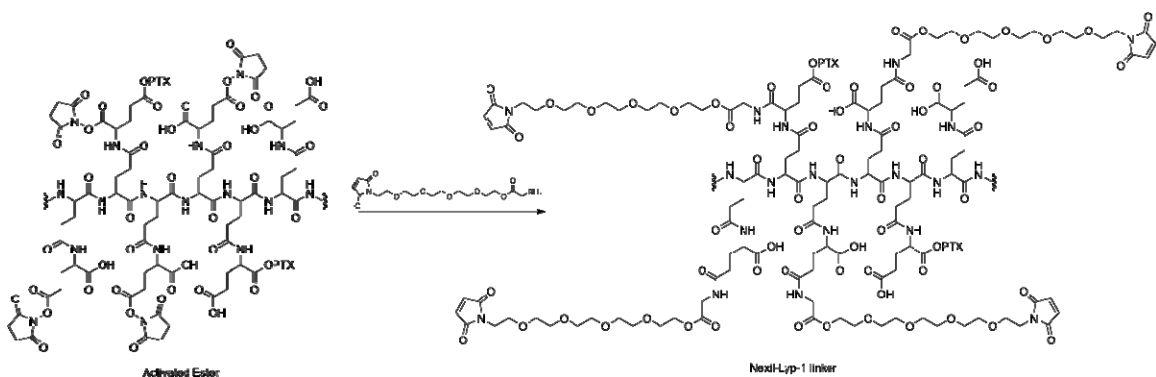
In a round bottom flask at room temperature and under argon atmosphere the material obtained in the previous reaction (activated Nexil) was dissolved in dry DMF and to this solution the Lyp-1 linker was added. It was calculated and excess of the linker to ensure the reaction completion; in this case the amount of linker was estimated for 45 units of 100mg of activated Nexil:

$n = 1.85 \times 10^{-6} \times 45$
$n = 8.32 \times 10^{-5}$
MW = 374.39
M = 31mg

Triethylamine
$n = 8.32 \times 10^{-5} \times 1.2$
MW = 101.19
d = 0.726
V = 14µL

The reaction was stirred at room temperature during approximately 36 hours. After this time the reaction was quenched adding cold Et$_2$O promoting the precipitation of the polymer. The suspension was transferred to a centrifuge tube and the sample was centrifuged at 10°C during 10 minutes at 3600 rpm. The solvent was removed and the precipitated was transferred to a round bottom flask and dry in high vacuum pump overnight. The ^{1}H NMR spectrum shows the characteristic peak of the maleimide group at 7.02 ppm apart from the resonance of the PEG protons between 3.4-3.7 ppm.

Second Step- Nexil-Lyp-1 linker peptide coupling reaction
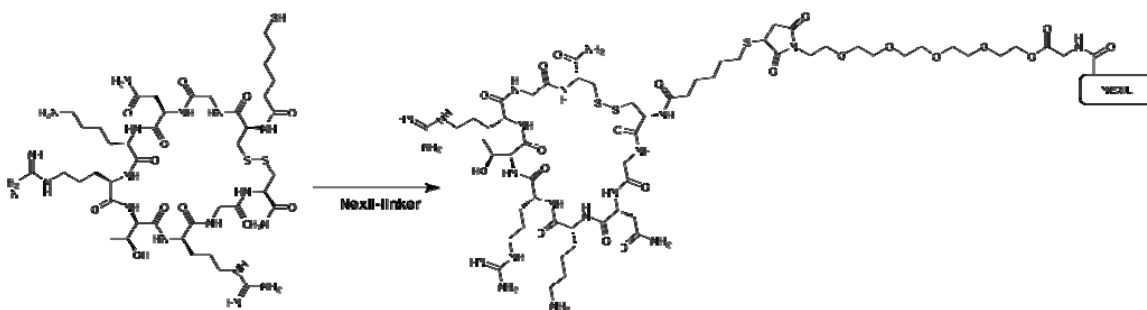
In a round bottom flask the Nexil-Lyp-1 linker (50 mg) was dissolved in DMSO (2 mL) and to this solution it was added PBS buffer pH 7 (1mL). The Lyp-1 peptide (50mg) was added to this solution and reaction was stirred overnight at room temperature. The reaction was quenched adding water and the solution became cloudy (it is very likely that the Nexil-Lyp-1 conjugate it is not enough hydrophilic to bring the compound into solution in the acidic form). The compound was transferred to a dialysis bag and the compound was dialyzed overnight. The compound was lyophilized and analyzed by NMR. A small sample of the compound obtained was dissolved in deuterated DMSO and ^{1}H NMR spectrum was taken. Unfortunately in this first attempt it was not possible to make sure the desired compound was obtained.

In order to optimize the reaction conditions for this coupling, it was tested a new conditions where only the Lyp-1 peptide and linker was reacted. The advantage of using these compounds is the simple analysis of the reaction outcome by analytical HPLC. The reaction conditions were similar to the one described previously, but instead of DMSO it was used acetonitrile. One aliquot from the reaction was taken after two hours and injected in the HPLC revealing the reaction completion. This has indicated that the "ideal" reaction conditions for the present coupling is mixture of acetonitrile:PBS buffer 7.0.

KEY RESEARCH ACCOMPLISHMENTS

- Further validated Nexil as a platform for drug delivery.
- Designed and synthesized two different linkers to be used in the coupling with the RGD peptide (PROPRIETARY).
- Synthesized cyclic RGD peptide using Fmoc strategy on solid support.
- Coupled cyclic RGD to linkers, deprotected and documented purity.
- Synthesized a set of molecules containing 5, 15 and 30 RGD units per polyglutamylglutamate molecule.
- Established fluorescence polarization assay for assessment of affinity of binding to soluble integrins.
- Added fluorochrome to RGD-linker-polymer to facilitate pharmacokinetic and tissue distribution studies.
- Synthesized Nexil-DTPA to permit loading with ^{99}TC or ^{111}In for both pharmacokinetic studies and external imaging of Nexil distribution.
- Established protocol for loading of Nexil-DTPA with ^{111}In.

- Conducted in vivo experiments documenting a high correlation between tumor uptake of Nexil and delivery of paclitaxel in a panel of 8 different human tumor xenograft models.
- Conducted in vivo experiments that determined the tumor and plasma pharmacokinetics of RGD-targeted Nexil in the H460 human tumor lung xenograft model
- Synthesized linkers for coupling Lyp-1 to Nexil
- Established a synthetic route for the production of Lyp-1
- Synthesized Lyp-1 coupled to two different length linkers
- Synthesized test batches of Lyp-1 coupled to Nexil

REPORTABLE OUTCOMES

Presentations

None

Abstracts

None

Manuscripts

None

CONCLUSIONS

We have established that there is an excellent correlation between the tumor uptake of Nexil and its ability to deliver paclitaxel in a panel of 8 human tumor xenografts. In addition to facilitating the pharmacokinetic analysis of various forms of peptide-target Nexil the results of these experiments provide a strong rationale for the development of Nexil-DTPA as an agent for selection of patients to be enrolled on Nexil clinical trials in the future.

We have successfully synthesized RGD-target Nexil and conducted and analysis of the dependence of its binding and toxicity to integrins using an in vitro model. Initial in vivo experiments using the H460 human lung cancer xenograft model directed at determining the tumor and plasma pharmacokinetics of RGD-targeted Nexil did not demonstrate a clear advantage for the RGD-targeting.

A great deal of effort has gone in to developing routes for the synthesis of Lyp-1, the linkers needed to couple it to Nexil and the Lyp-1-linker complex. We have finally succeeded in making small batches of the final product consisting of the Lyp-1-linker-Nexil complex. Next steps will include efforts to introduce a tritiated form of paclitaxel into this complex and then conduct tumor and plasma pharmacokinetic studies in the H460 model.

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22.   If the residue cannot be solublize in the mixture mentioned (MeOH:water). The solvent needs to be removed on the rotavaporator and the residue obtained should be wash it again with ether. This procedure needs to be done until the residue can be solubilized completely in the solvent mixture MeOH:water.

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